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14. ABSTRACT

The goals of this proposal are to: (i) identify prion protein (*Prnp*) variability in the Wisconsin free-ranging white-tailed deer population, (ii) determine the frequency of these *Prnp* alleles in CWD-positive and –negative deer, (iii) characterize the effect of these allelic variations on the biochemical and biological properties of the CWD agent and iv) to determine the likelihood of CWD transmission to humans. Deer *Prnp* alleles have been sequenced from CWD-positive and -negative deer. Several new alleles have been identified. Two alleles are underrepresented in the CWD-positive animals suggesting they may reduce susceptibility to infection. Tissue from genetically defined, CWD infected deer has been used for determining the protease digestion properties of the wild-type PrPCWD protein and for successfully orally infecting deer with CWD agent of known genotypes. Transgenic mice, expressing cervid PrP, have been successfully infected with CWD agent providing a rodent model for the analysis of CWD infection.

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Introduction:

CWD is the only prion disease occurring in wild, free-ranging animals (deer and elk). It has been identified in free-ranging cervids in Colorado, Nebraska, New Mexico, South Dakota, Saskatchewan, Wisconsin and Wyoming. In Wisconsin, this contagious disease is endemic in a region of the state having an exceedingly high deer density (~53 deer/square mile). It is not known whether all deer are equally susceptible to CWD. Since the prion protein gene is the genetic susceptibility factor for prion diseases, we initiated a study to define the prion protein gene in Wisconsin white-tailed deer. The identification of four different deer PrP proteins and their apparent unequal frequency in CWD infected and uninfected deer suggest there may be differing levels of genetic susceptibility to the disease. In addition, the existence of different PrP proteins raises the possibility of the existence of different CWD strains. Our proposed studies will focus upon characterizing the PrP gene in additional CWD-infected and uninfected deer, determining whether different CWD strains exist and the potential of the genetically defined CWD isolates to cause disease in humans.

Statement of Work:

Specific Aim 1. To determine the sequence of deer prion protein genes in CWD infected and uninfected deer.

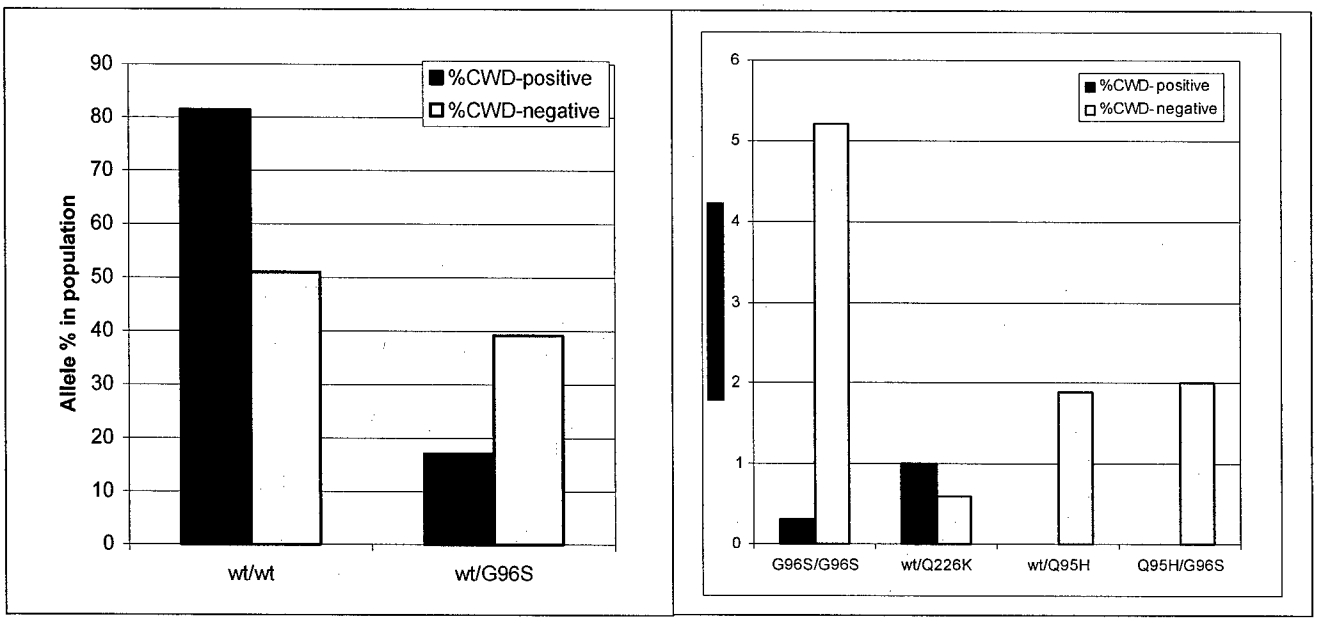
a. Isolate DNA from tissue samples of CWD infected and uninfected deer (Months 1-36).

Tissue samples were provided by the Wisconsin Department of Natural Resources and were obtained from deer harvested in Wisconsin. Muscle and/or ear tissues were collected from deer heads previously sampled for CWD by the Wisconsin DNR. Tissues have been collected from deer from the 2002-2004 hunting seasons. DNA was isolated as described in Johnson et al., 2003. From the Wisconsin CWD endemic region, samples from a total of 292 positive animals and 153 negative animals have been collected. Another 179 negative animals, from outside the CWD endemic region, have also been analyzed.

The initial samples were tested for were tested for the presence of PrP^{CWD} in the obex and retropharyngeal lymph nodes using immunohistochemistry (IHC) at the Wisconsin Veterinary Diagnostic Lab in Madison, Wisconsin. Deer defined as CWD-positive stained positive for PrP^{CWD} in either the obex and/or retropharyngeal lymph nodes. Deer defined as CWD-negative were animals that were obex and lymph node IHC-negative for proteinase K-resistant PrP. In subsequent years, different USDA-approved assays have been used by the WVDL for determining whether animals were positive for CWD, both the IDEXX test and the Bio-Rad test have been utilized.

b. PCR amplify and directly sequence the prion protein gene coding region (Months 1-36)

We have continued to use PCR and direct sequence analysis to determine the prion protein gene coding region from both infected and uninfected deer. Our initial study, examining 26 CWD-positive and 100 CWD-negative deer, identified 4 Prnp alleles. One of these alleles, QGS or wt, appeared to be underrepresented in the CWD-affected animals. To determine if there is a link between specific *Prnp* alleles and susceptibility to CWD, *Prnp* was sequenced from an additional



193 CWD-positive and 153 CWD-negative white-tailed deer from the CWD-affected region of

Figure 1. Analysis of white-tailed deer *Prnp* in Wisconsin. Four different PrP alleles were identified. The three amino acids listed with each allele represent the three variable positions at residues 95, 96 and 226. The amino acid change at position 138 is associated with the processed pseudogene. The percent of each allele in the respective populations is presented.

south-central Wisconsin. Approximately 98% of the functional *Prnp* genes in the CWD-negative white-tailed deer population are comprised of two major alleles, wt (71.9%) and G96S (25.8%). The *Prnp* genotypes present in the CWD-positive and -negative deer populations in the CWD-affected region of Wisconsin are significantly different from each other ($p<0.001$) (Fig. 1). In the CWD-positive deer population, 81.5% of the animals were wt/wt compared to 51% of the CWD-negative deer. The wt/wt genotype is significantly over-represented in the infected deer population ($p<0.0001$). Conversely, 17.4% of CWD-positive deer had at least one copy of the G96S allele, compared to 44.4% of CWD-negative deer. The wt/G96S and G96S/G96S genotypes represent 39.2% and 5.2% of the CWD-negative animals, respectively; however, significantly fewer CWD-positive deer were identified with these alleles (17.1%; $P<0.0001$ and 0.3%; $P=0.0005$, respectively) (Fig. 2). In addition to the G96S allele, deer heterozygous for the Q95H allele were also significantly under-represented in the CWD-positive population ($p=0.007$). No Q95H allele was identified in the CWD-positive deer population, while 3.9% of the CWD-negative deer assessed from the affected region were Q95H heterozygous. There was no significant difference between CWD-positive and CWD-negative deer for the presence of the pseudogene (20.9% and 18.3%, respectively; $p=0.5165$).

We previously estimated that approximately 91% of the CWD-negative deer had allelic combinations found in CWD-positive deer suggesting that there was not a complete barrier to transmission. By expanding the number of animals genotyped, we determined that 96+/-3 % of the white-tailed deer are genetically susceptible to CWD. Our studies indicate that, although

there are two alleles that confer levels of resistance to CWD, resistance is not complete and the alleles are not abundant. Thus, significant genetic barriers to disease progression do not exist in the Wisconsin white-tailed deer population.

Confirmation of S138N as a pseudogene: Two methods were employed to determine if the S138N change detected in the Wisconsin white-tailed deer population was a processed pseudogene. A step-down PCR genome walking protocol, described by Zhang & Gurr (2000), was adapted to characterize 5′- and 3′-flanking regions of the white-tailed deer *Prnp* open reading frame (ORF) (Figure 2). A shrimp alkaline phosphatase (SAP)-treated adaptor was ligated to SpeI, NheI or XbaI digested genomic DNA or a SAP-treated adaptor was ligated to XmaI or NgoMIV digested genomic DNA. Using a white-tailed deer *Prnp*- and an adaptor-specific primer, nested-PCR was subsequently performed to amplify 5′ and 3′ regions flanking the ORF. Primers specific to white-tailed deer *Prnp* are described in Table 1. PCR products were fractionated on 1% agarose gels and purified using Qiaquick gel purification kit (Qiagen, USA). Purified products were ligated into pGEM-T Easy vector (Promega, USA) and sequenced using T7- and SP6-specific primers (Promega, USA) as described previously (Johnson *et al.*, 2003).

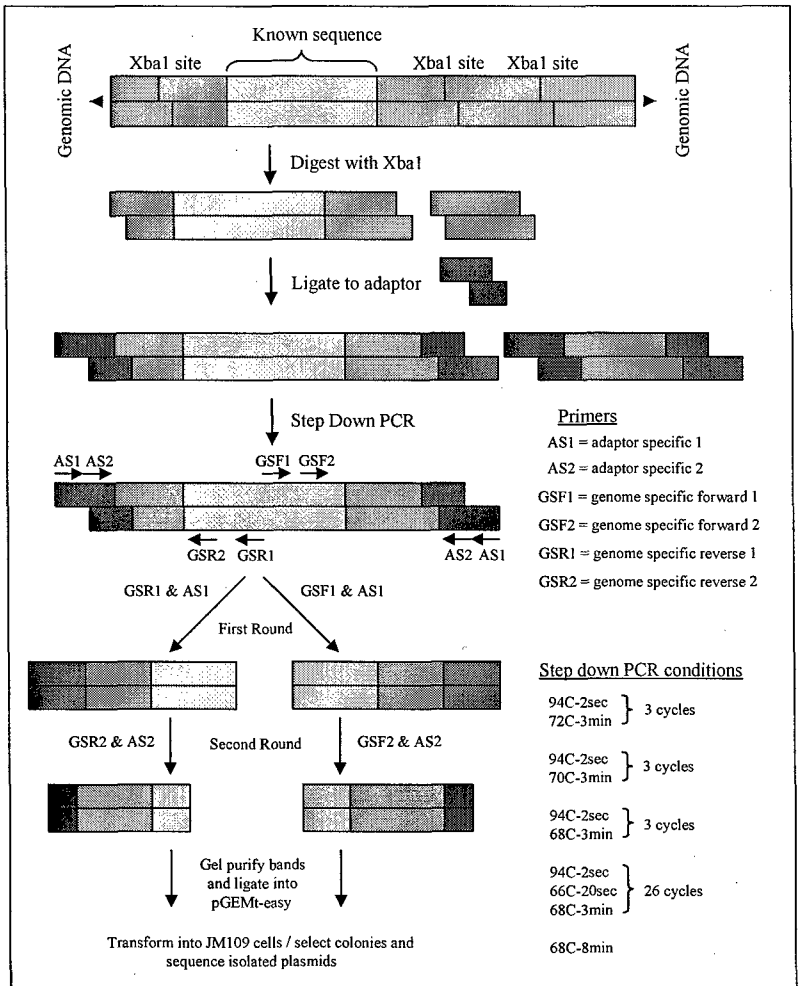


Figure 2. Schematic representation of "step down" genome walking.

The second approach involved cloning PCR amplified *Prnp* from deer identified, by direct sequencing, as heterozygous for G96S and S138N polymorphisms. The gene was amplified, cloned and sequenced as described above.

Three unique *Prnp* clones were isolated and characterized from a single animal: G96S, S138N, and wt indicating the presence of at least one additional copy of *Prnp*. The 5′-upstream region of *Prnp* was also sequenced. A BLAST search against GenBank, using *Prnp* sequence from the 5' untranslated region of the allele with the S138N change, showed 98% sequence similarity with the mule deer processed pseudogene (GenBank

accession # AY371694).

Table 1. Primers for genome walking:

Adaptor specific primers:
AS1=PP1(Tm 52.5°C) 5'-GTAATACGACTCACTATAGGGC-3'
AS2=PP2(Tm 59.5°C) 5'-ACTATAGGGCACGCGTGGT-3'

Genome specific primers:
GSF1=CervidF1(Tm 64°C) 5'-TGGGCCTCTGCAAGAAGCGACCAAAACCT-3'
GSF2=CervidF2(Tm 66 °C) 5'-GGCCTTGGTGGCTACATGCTGGGAAGTG-3'
GSR1=CervidR1(Tm 66°C) 5'-ATGAGGAAAGAGATGAGGAGGATCACAGGAGGG-3'
GSR2=CervidR2(Tm 67°C) 5'-GAAGTTCTCCCCCTTGGTGGTGGTGGTGAC-3'

Specific Aim 2. To biochemically characterize the CWD isolates.

a. Protease resistance properties of existing CWD isolates (Months 6-24)

The CWD status of the hunter harvested deer was determined by the WVDL as described above. We previously demonstrated that, as expected, that all deer that were positive for CWD had protease-resistant PrP, whereas uninfected samples exhibited no staining after digestion. A time course of PK digestion of brain homogenate from a CWD^+ deer with a wt/wt *Prnp* genotype indicates the presence of PK resistant protein after a 2 hour digestion. This pattern of proteinase K resistance is typical of many other TSEs.

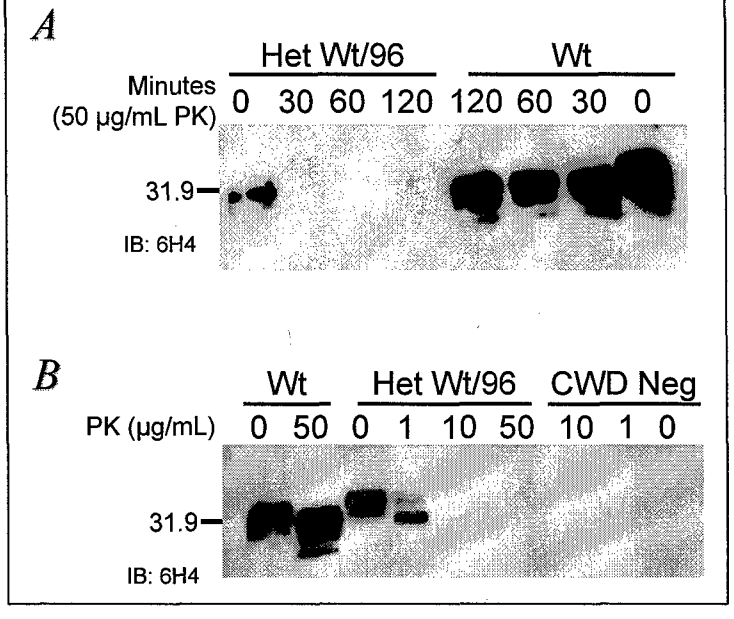


Figure 3. A)Time course of proteinase K digestion of PrPCWD from wt/wt and G66S heterozygous animals. B) PK concentration curves for wt/wt and wt/G96S PrPCWD. Note that the PrP from the heterozygous animal is much less resistant to PK.

We have characterized the degree of sensitivity to proteinase K digestion of brain tissue from wt/wt and wt/G96S heterozygous deer. PrP^{CWD} from wt/wt deer exhibit a typical pattern of resistance to protease digestion, with protein still remaining after 2 hours of digestion. Although we have brain tissue from several hunter-harvested wt/G96S deer, only two were obex-positive. Since neither sample was strongly positive, we have concentrated the PrP^{CWD} from both the wt/wt and the wt/G96S obex samples using phosphotungstate preci-

itation. As can be observed in Figure 3, the PrP^{CWD} present in the wt/G96S heterozygous animal is apparently much less resistant to protease digestion compared to PrPCWD from wt/wt animals. This suggests that the prion protein in the heterozygous animals has a different conformation and, perhaps, different properties than wt/wt animals. Only one wt/G96S heterozygote has been analyzed so it is premature to assume that different strains of CWD may exist. The data is, however, suggestive of the existence of another CWD strain and we will analyze other heterozygous animals as they become available. We have two sources of heterozygous animals: i) from hunter-harvested free-ranging deer and ii) the heterozygous animals that were orally infected with CWD (specific aim 3).

b. Protease resistance properties of new CWD isolates discovered in Specific Aim 1. (Months 36-54)

Not applicable

c. Cell-free conversion assays

We are continuing to develop the tools for the cell-free conversion assays to determine, in vitro, the ability of CWD agent from animals with different genotypes to convert PrP^{C} with specific primary amino acid sequences. We are anticipating, for example, that the 95 and 96 alleles (both underrepresented in the CWD-positive population) to be unable to be efficiently converted to the abnormal form of the protein in the presence of CWD from wt/wt animals. Several different approaches are being used to prepare PrP^{C}; baculovirus expression systems, mammalian expression systems and bacterial systems using biotin fusion.

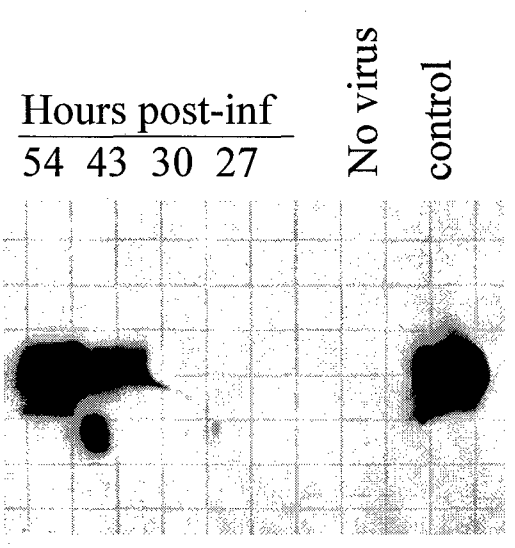


Figure 4. Western blot analysis of PrP expression in baculovirus cells. PrP is first expressed ~43 hours post-infection of the insect cells.

Initial isolation and amplification of whitetail PrP genes has already been completed for the WT and 96 alleles. These alleles were ligated into the multiple cloning site of pIREShyg at the Not1 restriction enzyme cut site. The insert fragments were then subcloned into the pVL1392 baculovirus transfer vector and transformed into bacteria. The insertion and sequence of the inserted DNA was then confirmed to be the correct PrP coding region by direct sequence analysis. Both the wt and G96S sequences have been cloned into the transfer vector. These clones have been co-transfect insect cell lines and we now have cloned baculoviral stocks for the cervid wild-type PrP. The expression of the cervid wild-type PrP has been confirmed by Western blot analysis (Figure 4). The next step is to generate radio-labelled PrP^{C} for use in the cell-free conversion reactions.

The G96S prion protein construct has been co-transfected into the Sf9 insect cell line and we have viral supernatants containing the G96S prion protein. We are in the process of cloning out the viral stocks to ensure pure baculovirus stocks.

We are also expressing cervid PrP in *E. coli* as a cleavable PrP/Biotin fusion protein (Pinpoint Expression). PrP/Biotin fusion proteins will be used in our cell-free conversion assays. The *Prnp* ORF was amplified for insertion into the pinpoint vector (neither the signal sequence for targeting the protein to the mammalian cell membrane or the GPI anchor signal sequence were included)l. Primers were designed to amplify the portion of *Prnp* encoding for amino acids (AA) 23-227. In doing so 6 AA were trimmed from the carboxy-terminus of the mature peptide, but only the predicted membrane signal sequence was omitted on the amino-terminal end. Two reverse primers were necessitated because the primer overlapped the codon for the Q226K cervid PrP allele. All primer sequence was complementary to target sequence except the addition of a stop codon on the reverse primers.

Primers:

PinF(Tm 60.6°C)	5'-CCTCTGCAAGAAGCGACCAAAACC-3'
PinR(Tm 57.8°C)	5'-TTAAGCCTGGGATTCTCTCTGGTAC-3'
PinR226(Tm 56.1°C)	5'-TTAAGCCTTGGATTCTCTCTGGTAC-3'

Prnp from Wt, Q95H, G96S and Q226K have been amplified, ligated into the Pinpoint vector (Promega), transformed into JM109 cells (Promega) and clonally selected for in frame orientation to the biotin signal. Clones were then grown in the presence of biotin and induced to express from the plasmid by the addition of IPTG. Four hours post-induction, cells were harvested and boiled with SDS-PAGE buffer. Western blot analysis was then performed using the 6H4 anti-PrP monoclonal antibody (Prionics) (Figure 5). All clones had reactive bands indicating the synthesis of recombinant PrP for each of the alleles. We are repeating the transformation of the Q95 H allele due to the low expression observed (Figure 5). In addition a putative new allele, an A123T change found in a deer from outside the endemic region, has been cloned into the pinpoint vector. There is no original tissue to reisolate genomic DNA from this individual animal to confirm the allele change, but direct sequencing identified the change and we have been able to clone it from the original *Prnp* PCR amplification.

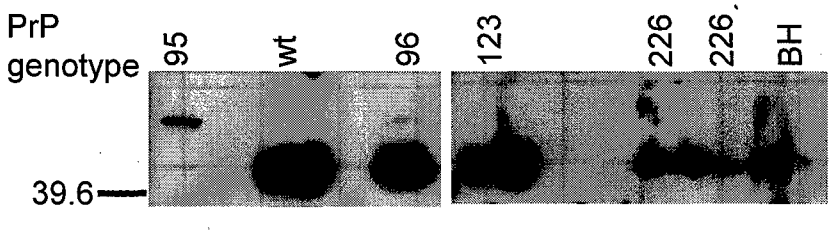


Figure 5. Western blot demonstrating the expression of cervid PrP in E.coli cells. Wt, G96S and Q226K are all expressed at relatively high levels. Expression of Q95H is low.

Specific Aim 3. To characterize the CWD isolates by transmission studies into deer and transgenic mice expressing deer PrP.

a. Identification of does and fawns of appropriate PrP genotype (Months 1-12)

Seventeen fawns were made available to us by the Northlands Deer Rehabilation Center in Minocqua, Wisconsin. Buccal swaps were taken from all animals and the PrP genotype determined as described in Johnson et al., 2003. After the fawns arrived in our facilities, genotyping was repeated to confirm our earlier analysis. PrP genotypes for each animal is presented in Table2. For the second set of Prnp genotyping experiments, testicular tissue and/or muscle tissue was used as the DNA source. In September of 2003, all deer were vaccinated, males were castrated and lymph biopsies taken from all animals. To confirm that all fawns, born in regions where CWD is not present, were indeed CWD-free, the lymph biopsies were analyzed, immunohistochemically, for PrP by the personnel at the Wisconsin Veterinary Diagnostic Laboratory. All animals were lymph node negative for PrP.

Deer #	Q95H	G96S	Wild type
1293			++
1277			++
1289			++
1256			+
1285		+	+
1258			++
1291			++
1260			++
1279	+		+
1275		+	+
1287			++
1295			++
1283		+	+
1297	+	+	
1281		+	+

Table 2. Genotype of Deer Experimentally Infected with CWD

b. Inoculation and incubation of CWD in deer. Three separate inoculations (Months 6-24 & Months 24-42, Months 42-60)

The first set of fawns were orally inoculated with brain homogenate from CWD+ deer with defined *Prnp* genotypes on May 11, 2004. Three animals served as uninfected controls (fawns 4, 6 and 8) and the remaining 12 animals were orally dosed with inocula from wt/wt CWD^+ deer. Inoculated animals received 20 ml of a 50:50 mixture of applesauce and 20% brain homogenate daily, for 5 days. At 460 days post-inoculation, 10/12 experimentally infected animals remain clinically normal. Two animals have been euthanized. One animal (animal #1283) was euthanized at 78 days post-inoculation due to a hindlimb absess. This animal was fully necropsied and tissues were frozen for subsequent analysis (described below). The second animal (animal #1287) was found dead 445 days post-inoculation. It was also fully necropsied and all tissues from this animal have been archived for subsequent analysis.

Initial characterization of animal 1283. Although this animal did not display any clinical symptoms of disease and the time of death was prior to any anticipated clinical disease, we tested the lymph nodes for the presence of CWD. Our colleague, Dr. Delwyn Keane at the WDVL, has previously demonstrated that positive staining for CWD first occurs in the retropharyngeal lymph nodes. As shown in Figure 6, the retropharyngeal lymph nodes do stain with antibodies

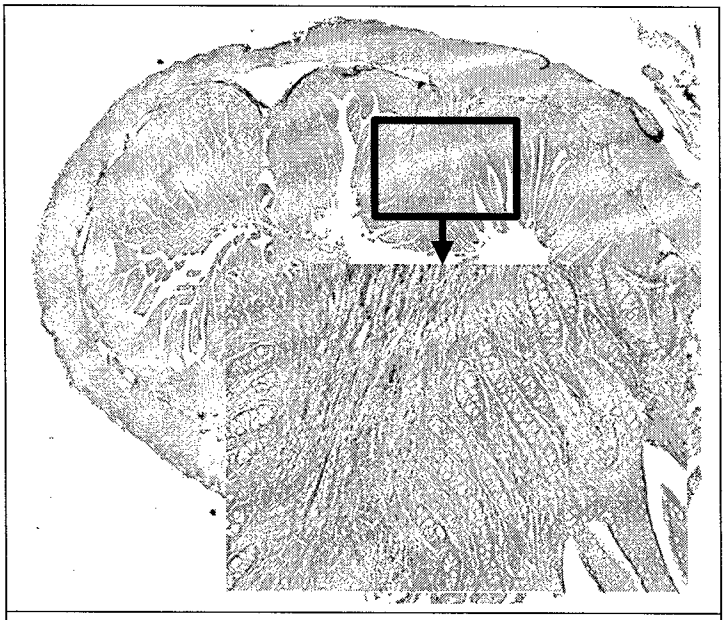


Figure 6. Immunohistochemical analysis of retropharyngeal lymph nodes from animal #1283. Red indicates the location of PrP^{CWD}.

against PrP indicating that the animal was, indeed, positive for CWD. This result confirms that we were successful in orally dosing our animals with chronic wasting disease. The low levels of PrP^{CWD} present in this animal was expected and reflects the early preclinical stages of CWD.

Initial characterization of animal 1287. This animal is a homozygous wt/wt animal (wrt *Prnp* genotype). As mentioned above, the animal dies 445 days (~13.5 months post-infection). Both the lymph nodes and the obex of this animal were analyzed via immunohistochemistry for the presence of PrP^{CWD}. In the retropharyngeal lymph nodes (Figure 7), all germinal centers contain PrP^{CWD}. The PrP^{CWD} deposition in the obex of the brain is analogous to stage 3 staining, i.e., heavily positive. Although this animal was not displaying overt clinical signs, differences in behavior had been noted (particularly an arched back, a

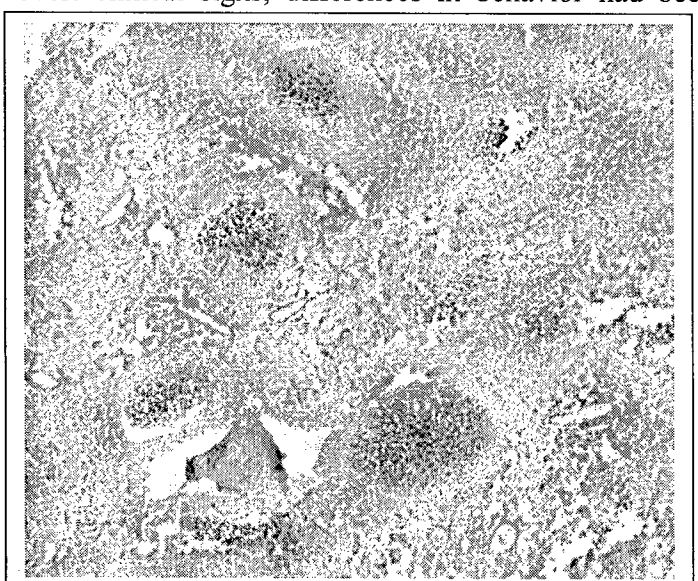


Figure 7. PrP^{CWD} deposition in the retropharyngeal lymph nodes of animal #1287. Note that PrP^{CWD} is present in all germinal centers.

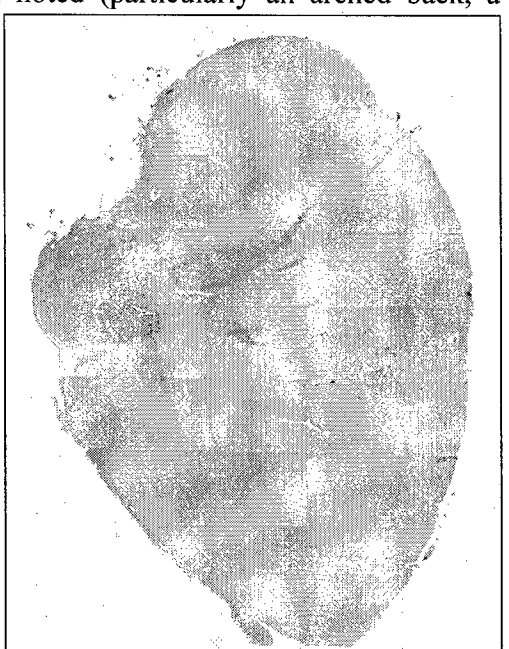


Figure 8. Deposition of PrP^{CWD} in the brain of animal #1287.

characteristic behavior observed in other TSE diseases). The animal had also been anesthetized the previous week for routine sample collection, it has been observed with experimental models of TSE infection that immediately prior to onset of clinical disease, an increased sensitivity to a variety of environmental insults. The distribution of PrP^{CWD} in this animal will be more fully analyzed in the next month, the animal died less than 2 weeks prior to the writing of this update.

c. Generation and Infection of transgenic mice

To study the effects of the elk codon 132 polymorphism, we generated transgenic mice expressing CerPrP in which we altered the expression of methionine at codon 132 to leucine by PCR site-directed mutagenesis. Transgenic lines referred to as Tg(CerPrP L132)1970 and Tg(CerPrP L132)1973 expressed cervid PrP with leucine at residue 132 (CerPrP-L132) at levels 3-fold higher than PrP in the brains of wild type mice while Tg(CerPrP-L132)1972 expressed PrP 16-fold over wild type levels. Tg(CerPrP L132)1972 mice became hyperactive at approximately 6 weeks of age which increased in severity until the mice became increasingly difficult to breed. Tg(CerPrP-L132)1972 mice were, therefore, not used in any studies.

Tg(CerPrP-L132)1970 and Tg(CerPrP-L132)1973 mice were inoculated with several CWD-positive mule deer or elk brain extracts. While these isolates caused disease in Tg(CerPrP) mice expressing methionine at codon 132, Tg(CerPrP L132) mice inoculated with CWD failed to develop symptoms associated with prion disease. Inoculated Tg(CerPrP L132) mice culled at 597 days post inoculation showed no clinical signs of prion disease and western blot analysis failed to detect PK-resistant PrP. These results suggest a protective effect of leucine at codon 132. Experiments designed to assess whether subclinical prion replication occurred in these inoculated mice are ongoing. Also, Tg(CerPrP, L132) mice have been inoculated with materials from sick elk that were homozygous L/L or M/L at residue 132.

Transgenic mice expressing cervid PrP with other polymorphisms have also been generated. Cervid PrP in which an additional octapeptide repeat region had been inserted, called CerPrP-ORI, has been used in the development of two Tg(CerPrP-ORI) mouse lines, Tg3879 and Tg3881. Tg(CerPrP-ORI)3879 mice express PrP at 2-fold over wild type levels and Tg(CerPrP-ORI)3881 express PrP 4-fold over wild type. Unfortunately, microinjections with CerPrP containing the serine polymorphism at codon 96, called CerPrP-S96, have not yet succeeded in generating a transgenic line with useful levels of PrP expression. Since N138S had been reported to be a polymorphic codon of mule deer and white-tailed deer, we designed two transgenic lines that expressed CerPrP with N138 instead of S138. More recently, the 138 codon has been demonstrated to be a processed pseudogene. Transgenic lines with CerPrP N138 were, therefore, not used in any studies.

We have also generated transgenic mice expressing bovine PrP with 5 or 6 octapeptide repeat regions, called Tg(B5) and Tg(B6), respectively. Transgenic mice expressing sheep PrP with various alleles of ARQ, ARR, AHQ, and VRQ polymorphisms are also in various stages of production. Studies of CWD susceptibility in these mice will facilitate studies on the origins of CWD and access the risk that CWD prions pose to livestock.

Specific Aim 4. To ascertain whether CWD is potentially transmissible to humans.

Transgenic animals expressing the human PrP gene at high levels is currently be developed. Only recently has tissue from one of the experimentally-infected animals become available. This tissue will be used to infect mice transgenic with the human PrP gene.

Key Research Accomplishments:

1. **Our genotyping of deer in the disease eradication zone of south-central Wisconsin suggests that deer with theG96S genotype have a reduced susceptibility to CWD.**
2. **Deer with the Q95H *Prnp* genotype may also have a reduced susceptibility to CWD.**
3. **We determined that 96+/-3 % of the white-tailed deer, in Wisconsin, are genetically susceptible to CWD.**
4. **The CWD pseudogene, described in mule deer and in captive white-tailed deer, is also present in the free-ranging white-tailed deer in Wisconsin.**
5. **Analysis of a deer with intercurrent disease demonstrates the presence of PrP^{CWD} in the retropharyngeal lymph nodes, confirming that the orally dosed animals were successfully infected with CWD.**
6. **An experimentally infected wt/wt deer was positive for CWD, with high levels of PrP^{CWD} deposition in the retropharyngeal lymph nodes and the obex of the brain.**
7. **Tg mice have been generated that will support infection with CWD agent.**

Reportable Outcomes:

Johnson, C., J. Johnson, J. Vanderloo, D. Keane, M. Clayton, J. Aiken and D. McKenzie.2005. Prion protein polymorphisms in the Wisconsin white-tailed deer herd influence susceptibility to chronic wasting disease (CWD). Keystone Symposia: Molecular Mechanisms of Transmissible Spongiform Encephalopathies (Prion Diseases).

Johnson, C., J. Johnson, D. Keane, J. Vanderloo, P. Bochsler, J. Aiken and D. McKenzie. 2005. Prion protein allele may provide resistance to CWD in Wisconsin white-tailed. Deer. 2nd International Symposium on The New Prion Biology: Basic Science, Diagnosis and Therapy, Venice, Italy.

Aiken, J., C. Johnson, J. Johnson, D. Keane, J. Vanderloo, P. Bochsler and D. McKenzie. 2005. The Second International Chronic Wasting Disease Symposium. Madison, WI

Johnson, C., J. Johnson, J.P. Vanderloo, D. Keane, J.M. Aiken and D. McKenzie. Prion Protein Polymorphisms in White-tailed Deer Influence Susceptibility to Chronic Wasting Disease, manuscript to be submitted by August 30, 2005.

Conclusions: All aspects of this project are progressing well. Analysis of two deer that were orally infected with CWD agent demonstrates that the oral infections were successful. One of the deer, euthanized prior to onset of clinical disease was positive for CWD in the

retropharyngeal lymph nodes, one of the first tissues to be positive in infection. The second animal, much further into the incubation period, had high levels of PrP^{CWD} in both the lymph nodes and the obex of the brain. These animals will provide tissue for subsequent inoculations as well as for biochemical analyses. We have sequenced a sufficiently large number of deer, both CWD-positive and –negative, to provide statistical power to our conclusions. The deer genotyping studies also suggest that the amino acid changes at codons 95 and 96 may affect susceptibility of deer to infection with the CWD agent. Subsequent inoculations into deer will further substantiate this observation. Mice, carrying the cervid *Prnp* transgenes, can be successfully infected with the CWD agent. These transgenic mice will provide an alternative model for testing strain differences in CWD inocula derived from deer with different *Prnp* genotypes.